PATENT
Docket No.: 19603/3232 (CRF D-2587B)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Goldman et al.	Examiner: Q. Nguyen
Serial No.	:	09/846,588	
Cnfrm. No.	:	4784	Art Unit: 1636
Filed	:	May 1, 2001	
For	:	METHOD OF INDUCING NEURONAL PRODUCTION IN THE BRAIN AND SPINAL CORD	

THIRD DECLARATION OF STEVEN A. GOLDMAN, M.D., Ph.D. UNDER 37 C.F.R. §1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, STEVEN A. GOLDMAN, pursuant to 37 C.F.R. § 1.132, declare:

1. I received B.A. degrees in Biology and Psychology from the University of Pennsylvania in 1978, a Ph.D. degree in Neurobiology from Rockefeller University in 1983, and an M.D. degree from Cornell University Medical College in 1984.

2. I am a Professor and Chief, Division of Cell and Gene Therapy, Glenn-Zutes Chair in Biology of the Aging Brain, Department of Neurology, University of Rochester Medical Center, Rochester, New York.

3. I am a named inventor of the above patent application.

4. I am the same Steven A. Goldman who signed the Second Declaration of Steven A. Goldman Under 37 C.F.R. 1.132 ("Second Goldman Declaration") in connection with my above application and am submitting the current declaration to present additional data relating to my invention.

5. Students in my laboratory working under my direction constructed replication-incompetent AdBDNF, AdNoggin, and AdNull adenoviral vectors and injected them

intraventricularly into Huntington mutant R6/2 mice and into normal wild-type mice, as described in paragraphs 4 to 7 of the Second Goldman Declaration.

Effect of AdBDNF Alone and AdBDNF/AdNoggin Delayed Functional Deterioration

6. Motor coordination and balance were measured using rotarod analysis (Andreassen et al., *Neurobiol Dis* 8:479 (2001)). AdBDNF/AdNoggin (n=10), AdBDNF (n=17) and AdNull (n=19) treated R6/2 mice were assessed by rotarod, as were untreated controls (n-17), beginning at 4 weeks of age. The mice were trained three times daily for two consecutive days on a rotarod (7650, UGO basile, Biological Research Apparatus, VA, Italy), at a constant speed of 12 rpm; they were subsequently tested weekly at the same speed. At each weekly test, each mouse was given three trials on the rod, and their latencies to fall measured. A maximum latency was defined at 300 sec., at which the individual test was terminated and scored as 300 sec.; for every 3-trial test, the best result, i.e., the longest time spent on the rod without falling, was recorded. All mice were tested from the day before stereotaxic surgery, at 4 weeks of age, until either 13 weeks of age, or until they were unable to maintain their body posture, whichever was later. Rotarod scores of <60 sec were considered neurologically abnormal (Laforet et al., *J. Neurosci* 21:9112 (2001)). This criterion was used to define impairment, the incidence of which was evaluated weekly. Comparisons of the mean duration of rotarod performance as a function of age were performed by ANOVA, followed by post-hoc Bonferroni t-tests.

7. To assess the effect of AdBDNF/AdNoggin-injection upon the functional deterioration of R6/2 mice, both rotarod testing and open-field analysis of volitional locomotion were used. It was also examined whether AdBDNF/AdNoggin co-treatment, which yields substantially more neuronal recruitment than that afforded by AdBDNF alone, resulted in better motor performance than that achieved solely by BDNF (Canals et al., *J. Neuroscience* 24:7727 (2004)). All mice were trained on the rod by 4 weeks of age, and then tested weekly at a constant 12 rpm (Andreassen et al., *Neurobiol Dis* 8:479 (2001)). It was found that the AdBDNF/AdNoggin-treated mice exhibited a significant deceleration in motor deterioration, relative to AdNull-treated R6/2 controls (attached Figure 1A). When the latency to fall off the rotarod (y) was plotted as a function of post-operative survival (m), curves were generated for AdBDNF/AdNoggin- and AdNull-injected mice that appeared to diverge at approximately 5 weeks after treatment. Simple regression analysis revealed that whereas the motor performance of AdBDNF/AdNoggin-treated animals could be described by the line $y = -28.5x + 321.6$, that of their AdNull-treated controls was described by $y = 44.7x + 318.2$. ANOVA of these regressions

revealed that the rate of deterioration of motor performance, as reflected in the regression slopes, was significantly influenced by treatment ($F=4.68$ [3, 68 df]; $p=0.005$). Post hoc analysis showed that the rate of motor deterioration was significantly greater in AdNull-injected R6/2 mice than in either their AdBDNF/AdNoggin or AdBDNF-treated counterparts ($p=0.008$ and 0.024, respectively).

8. When rotarod performance was compared between groups at each time point, again using ANOVA with post hoc tests, it was found that by 7 weeks after treatment, the AdBDNF/AdNoggin-injected R6/2s performed significantly better than their AdNull and untreated controls ($p=0.007$ and 0.012, respectively). In addition, the AdBDNF/AdNoggin-treated R6/2s exhibited a performance advantage over mice treated only with AdBDNF, which achieved statistical significance by 9 weeks post-treatment, at 13 weeks of age ($p=0.003$) (attached Figure 1A). When assessed with regard to their ability to sustain 60 sec of rotarod performance, the AdBDNF/AdNoggin-treated R6/2 mice performed significantly better than their controls by 11 weeks of age, or 7 weeks after viral injection ($p < 0.001$; $F=6.14$ [3, 52 df]). This difference was sustained through 13 weeks of age ($p = 0.001$; $F=6.55$ [3, 32 df]); post hoc comparisons thereafter became difficult as the control animals died, yielding a disproportionate representation of AdBDNF/AdNoggin-treated animals; rotarod testing was halted at that point. Similarly, open-field testing revealed that net locomotion was relatively preserved in AdBDNF/AdNoggin-treated R6/2 mice, which exhibited significantly more volitional horizontal explorative behavior than either their null controls or AdBDNF-treated mice at 13 weeks of age ($p=0.012$; $F=4.39$ [3, 29 df]) (attached Figure 1B).

9. Importantly, the increments in both 60 sec rotarod or open-field performance measures noted in AdBDNF/AdNoggin-treated mice were not replicated by AdBDNF alone (attached Figures 1A-1B). To the contrary, AdBDNF treatment alone never yielded a significant increment in either measure, relative to AdNull or untreated R6/2 controls. These data suggest that the robust neuronal recruitment associated with AdBDNF/AdNoggin correlated with functional improvement, while the more limited neurogenic and neurotrophic effects of BDNF alone failed to do so.

Effect of AdBDNF Alone and AdBDNF/AdNoggin on Survival

10. AdBDNF/AdNoggin, AdBDNF and AdNull-treated R6/2 mice were assessed for viability twice daily beginning at 4 weeks of age. Uninjected R6/2 mice were also included as untreated negative controls. To exclude the possibility that net survival might be

affected by rotarod or open-field testing, the mice in the survival study were not subjected to any behavioral assessment. Survival data were analyzed by Kaplan-Meier survival curves.

11. In light of the motor performance increments noted in AdBDNF/AdNoggin-treated R6/2 mice, it was examined if treatment influenced survival. In addition, it was examined if the effects of AdBDNF/Noggin co-treatment were significantly better than those achieved with BDNF alone. To this end, the mean survival of matched groups of R6/2 mice (n=10/group) were compared after being treated at 4 weeks of age with either: 1) a single AdBDNF/Noggin injection; 2) AdBDNF alone; 3) AdNull:GFP control; or 4) no treatment at all. The mice were then returned to their cages and followed, with supportive husbandry until death. It was found that AdBDNF/AdNoggin co-injected R6/2 mice survived significantly longer than both AdBDNF-treated and untreated controls. Moreover, the net survival of AdBDNF-treated R6/2 mice treated with AdBDNF alone was no different than that of their AdNull-treated controls (Figure 1C). Kaplan-Meier survival analysis by SSPS revealed that AdBDNF/AdNoggin-treated R6/2 mice survived a mean of 110.0 ± 3.3 days, whereas AdNull and untreated controls survived 94.5 ± 3.2 and 96.6 ± 2.7 days, respectively. ANOVA revealed that the overall effect of treatment upon survival was significant ($p=0.011$; $F=4.45$ [3, 38 df]). Post hoc analysis confirmed the difference in mean survival between AdBDNF/Noggin-treated R6/2s and their AdNull and untreated controls ($p<0.01$ and <0.05 respectively), such that AdBDNF/Noggin-treated R6/2s survived an average of 16.8% longer than their AdNull controls. Although the effect of treatment achieved statistical significance at day 98 ($p=0.013$), it was due largely to the relative survival of AdBDNF/AdNoggin-treated mice; R6/2s treated only with AdBDNF survived 102.0 ± 2.2 days, which was not significantly better than AdNull and untreated R6/2 controls. Thus, the survival benefit associated with AdBDNF/AdNoggin treatment was not provided by AdBDNF alone (Figure 1C).

12. In the above experiments, we first established that adenoviral overexpression of BDNF induces neuronal recruitment to the neostriatum, from ventricular zone progenitor cells. These new neurons invade the bulk of the neostriatum, and differentiate largely if not exclusively as medium spiny neurons (MSNs). These new MSNs integrate into the normal striatal neuronal architecture, where they extend fibers to the globus pallidus, survive, and persist. We also found that the adult R6-2 HD mouse, a well-established and broadly accepted mouse model of Huntington's Disease, similarly retains a population of dividing subependymal progenitor cells in the striatal wall, and that these cells respond to BDNF overexpression in the HD mouse just as they do in the normal rat, by

generating neurons that depart the subependyma, and enter the striatum to integrate as medium spiny striopallidal neurons. We then found that when the enabling neurogenic effects of BDNF are supplemented by a concurrent suppression of gliogenesis, in the present case using noggin, an antagonist of the pro-gliogenic bone morphogenetic proteins, the number of new neurons added to the treated subject's neostriatum is significantly and substantially increased. This increase proved sufficient to improve both the motor performance and survival of AdBDNF/AdNoggin-co-treated R6-2 mice, relative to their untreated R6-2 controls. I believe that these findings offer great promise for using neuronal induction from endogenous progenitor cells as a feasible and effective therapeutic strategy in Huntington's Disease, a disease that is otherwise presently untreatable and both inevitably and rapidly fatal.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 12-2-04

Steven A. Goldman, M.D., Ph.D.

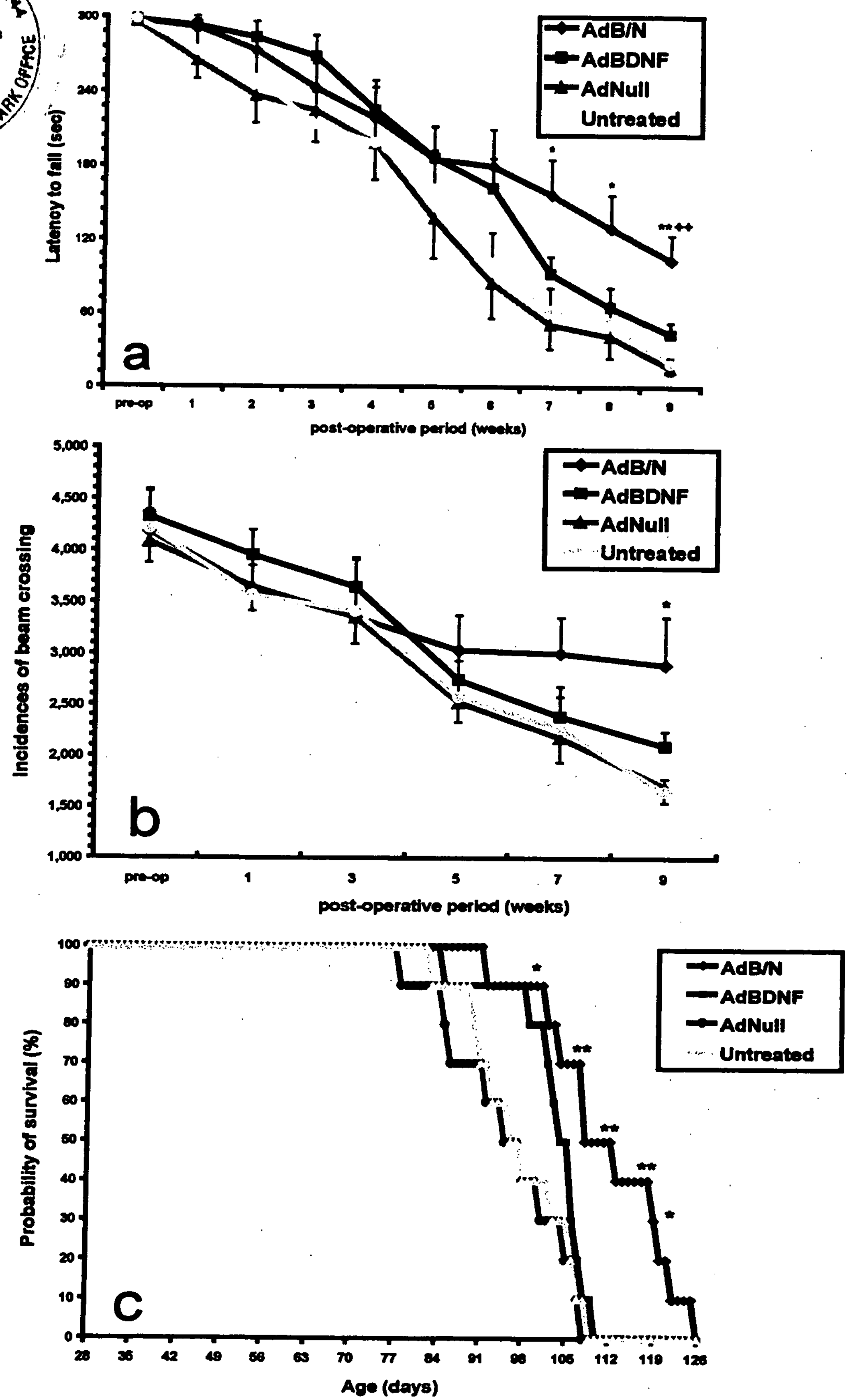
AdB/N
AdBDNF
AdNull
Untreated
Latency to fall (sec)
300
240
180
120
60
0
pre-op
1
2
3
4
5
6
7
8
9
post-operative period (weeks)
a
Incidences of beam crossing
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
pre-op
1
3
5
7
9
post-operative period (weeks)
b
Probability of survival (%)
100
90
80
70
60
50
40
30
20
10
0
28
35
42
49
56
63
70
77
84
91
98
105
112
119
126
Age (days)
c

Figure 1

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I, STEVEN A GOLDMAN, M.D., Ph.D., pursuant to 37 C.F.R. § 1.132, hereby declare:

1. I received B.A. degrees in Biology and Psychology from the University of Pennsylvania in 1978, a Ph.D. degree in Neurobiology from Rockefeller University in 1983, and an M.D. degree from Cornell University Medical College in 1984.

2. I am a Professor and Chief, Division of Cell and Gene Therapy, Glenn-Zutes Chair in Biology of the Aging Brain, Department of Neurology, University of Rochester Medical Center, Rochester, New York.

3. I am a co-inventor of the above-identified application, along with Abdellatif Benraiss.

4. I am a coauthor with Kim Lerner, Eva Chmielnicki, Neil Hackett, and Ronald Chrystal on the publication entitled "*In Vivo* Transduction of the Adult Rat Ventricular

Zone with An Adenoviral BDNF Vector Increases Neuronal Production and Recruitment to the Olfactory Bulb", Soc. Neurosci. 25: 413.3 (1999).

5. Kim Lerner and Eva Chmielnicki worked in my laboratory and performed various experiments at the instruction of Abdellatif Benraiss and me. They did not contribute to the conception of the invention as described and claimed in the above-identified application.

6. Neil Hackett worked in the laboratory of Ronald Chrystal and provided instruction to Abdellatif Benraiss on the preparation of adenoviral vectors. Ronald Chrystal and Neil Hackett did not contribute to the conception of the invention as described and claimed in the above-identified application.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 12/2/04, 2004

Steven A. Goldman, M.D., Ph.D.